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Modulation of oxidative stress by γ-glutamylcysteine (GGC) and conjugated linoleic acid (CLA) isomer mixture in human umbilical vein endothelial cells

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ABSTRACT

Individually, γ-glutamylcysteine (GGC), a dipeptide and precursor of glutathione (GSH), and conjugated linoleic acid (CLA), a trans-fatty acid, exhibit antioxidant properties. The objective of this study was to compare effects of co-administration of GGC and CLA to treatment with GGC alone on oxidative stress and GSH synthesis in human endothelial cells. Changes in levels of 8-epi-PGF2α, thiobarbituric acid reactive substances (TBARS), GSH, total antioxidants, GSH synthetase (GSS) expression, and transcription factor DNA binding were assessed in human umbilical vein endothelial cells (HUVEC) treated with GGC alone (100 μmol/L) or combined with CLA isomer mixture (10, 50, 100 μmol/L) for 24 h. Significantly higher levels of TBARS, 8-epi-PGF2α, GSH, and GSS protein were found in cells treated with GGC and 10 μmol/L CLA, compared to cells treated with GGC alone, indicative of prooxidant effects of CLA. Approximately 40% cell death was microscopically observed in cells incubated with GGC and 100 μmol/L CLA. Despite lower levels of GSH, treatment with GGC and 50 μmol/L CLA appeared to be protective from oxidative stress similar to treatment with GGC alone, as indicated by lower levels of TBARS, compared to control cells not treated with GGC and CLA.

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1. Introduction

Oxidative stress is associated with various clinical conditions (e.g., ischemia–reperfusion injury) and chronic diseases (Granger and Korthuis, 1995; Li and Jackson, 2002; Willcox et al., 2004). Glutathione (GSH) is the prevalent thiol-containing tripeptide antioxidant in mammalian cellular systems, intracellularly present at millimolar concentrations (Glantzounis et al., 2006; Franco et al., 2007). Increasing GSH levels could be beneficial for modulating oxidative stress-related injuries, diseases, and aging (Liu and Choi, 2000; Wu et al., 2004; Zeevalk et al., 2008). γ-Glutamylcysteine (GGC) is a dipeptide and precursor of GSH. GGC is synthesized by catalytic activity of GGC synthetase (GCS) from glutamate and cysteine. GSH is subsequently produced by the activity of GSH synthetase (GSS) from GGC and glycine (Franco et al., 2007). In healthy humans, intracellular (erythrocytes) and extracellular (plasma) GGC levels are approximately 66 and 4 μmol/L, respectively (Hagenfeldt et al., 1978). Unlike GSH, GGC uptake is not limited by plasma membranes or the blood brain barrier, and supplemental GGC can be directly used as a substrate for GSH synthesis (Dringen et al., 1997). Peptides with up to 51 amino acids, perhaps including GGC, can be taken up intact through plasma membranes via Na^+-coupled peptide transporter 1 (PEPT1) and transporter 2 (PEPT2) in various tissues (Rubio-Aliaga et al., 2003; Zhou et al., 2012; Chothe et al., 2011). In our recent GGC study (Nakamura et al., 2012), GGC appears to protect against oxidative stress by serving as a substitute for antioxidant GSH due to a SH group in its structure and modulating GSH synthesis.

Conjugated linoleic acid (CLA) has been reported to exhibit health promoting properties, such as anti-obesity, anti-carcinogenic, anti-inflammatory, and anti-atherogenic effects (Belury, 2002; Nakamura and Omaye, 2008; Kennedy et al., 2010; Gebauer et al., 2011). Previous studies have indicated that co-administration of nutraceuticals such as CLA with pharmaceuticals can augment the effects of the individual compounds. For instance, co-administration of CLA with a drug such as rosiglitazone or addition of resveratrol to the trans-10, cis-12 CLA isomer attenuates adverse effects associated with each compound (Liu et al., 2007; Kennedy et al., 2009; Halade et al., 2010). In addition, CLA can modulate oxidative stress by up-regulating GGC synthetase catalytic unit (GCS-HC) and subsequent GSH synthesis (Arab et al., 2006). Both GGC and CLA exhibit antioxidant properties. The heterogeneous nature of diets provided an environment for various interactions and relationships between endogenous/exogenous dietary substances. Our current interest is to establish a better understanding of such interactions and the subsequent effects on the antioxidant capacity of mixtures, including concentration dependent effects. These were thought to be timely studies because of the interest in the effects

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in co-administration of multiple pharmaceuticals. In addition, it is crucial to evaluate the concept that each chemical has an optimal concentration range for beneficial effects with possible detrimental effects beyond such range. The objectives of this study were to investigate a synergistic antioxidant role of CLA as an adjuvant and the effects of mixtures of compounds/co-administration seeking an optimal concentration range for beneficial effects by comparing co-administration of GGC and CLA to treatment with GGC alone on oxidative stress and GSH synthesis in human endothelial cells. Since CLA-induced adverse effects, such as increases in insulin resistance and inflammation, have been observed mainly by use of single purified CLA isomer (in particular the trans-10, cis-12-CLA isomer, but not the cis-9, trans-11-CLA isomer) (Halade et al., 2010; Kennedy et al., 2010; Martinez et al., 2010), a mixture of CLA isomers was chosen in this study. We assessed changes in levels of 8-epi-$PGF_{2\alpha}$, thiobarbituric acid reactive substances (TBARS), GSH, total antioxidants, GSS expression, and PPARγ and NF-κB DNA binding in human umbilical vein endothelial cells (HUVEC) treated with GGC alone (100 μmol/L: constant) or GGC together with CLA (the cis-9, trans-11 and trans-10, cis-12 CLA isomer mixture; 50% each) at graded concentrations.

2. Materials and methods

2.1. Chemicals and reagents

GGC was purchased from Bachem (Torrance, CA, USA). EGM Complete Medium (#CC-3024), HEPES Buffered Saline, and Subculture Reagents were purchased from Lonza (Walkersville, MD, USA). Power SYBR® Green Cells-to-CT™ Kit, Synth-a-Freeze, and PCR primers were purchased from Invitrogen (Carlsbad, CA, USA). CLA isomers (the cis-9, trans-11- and trans-10, cis-12-CLA isomer mixture), Nuclear Extraction Kit, PPARγ and NF-κB (human p50/p65 combo) Transcription Factor Assay Kits, Antioxidant Assay Kit, Glutathione Assay Kit, and 8-Isoprostane EIA Kit were purchased from Cayman Chemical (Ann Arbor, MI, USA). Primary antibody for human GSS was purchased from Abcam (Cambridge, MA, USA). Gelatin, o-phenylenediaminedihydrochloride tablets (SIGMAFAST OPD), and ExtrAvidin Peroxidase Staining Kit (EXTRA3) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVEC) were chosen in this study, since these cells are commonly used for investigations of molecule transport. HUVEC (#CC-2517) cryogenically preserved were purchased from Lonza. Cells were grown in the EGM Complete Medium containing fetal bovine albumin (2% final concentration) and all necessary growth factors, cytokines, and other supplements for cell growth/survival. Cells were subcultured by trypsin on 75 cm^2 gelatin-coated flasks and maintained at 37 °C in a humidified atmosphere of 5% CO_2 until becoming confluent. For RNA isolation and quantitative real-time PCR (qRT-PCR) analysis, cells were subcultured on a 96-well gelatin-coated plate.

2.3. Cell treatments and viability

HUVEC were grown on 75 cm^2 gelatin-coated flasks or 96-well gelatin-coated plate, and approximately ⩾95% confluent cells (~10^7 cells) were treated with GGC alone (100 μmol/L), GGC (100 μmol/L: constant) and CLA (the cis-9, trans-11 and trans-10, cis-12 CLA isomer mixture; 50% each; 0, 10, 50, 100 μmol/L), or not treated with GGC and CLA (control) for 24 h at 37 °C in a humidified atmosphere with 5% CO_2 (two flasks per each treatment for nuclear fraction collection; two 96-wells per each treatment for mRNA isolation; five flasks per each treatment for cellular fraction collection). The single concentration of GGC chosen (100 μmol/L) for each treatment group was the minimum concentration to significantly reduce levels of oxidative stress shown in our previous GGC study (Nakamura et al., 2012). After treatments for 24 h, cell viability was assessed microscopically. The sixth to ninth passages of tightly confluent mono-layered cells were collected after each treatment and used for subsequent analyses.

2.4. Cytoplasmic fraction preparation

After 24-h incubation with GGC alone or together with CLA, cells were rinsed, scraped, and suspended into ice-cold PBS (pH 7.4, 10 mmol/L of phosphate buffered saline, 138 mmol/L of NaCl, 2.7 mmol/L of KCl). Cells were collected from five 75 cm^2 flasks per each treatment and pooled. Cells were homogenized for 15 s at the maximum speed (Tissue Tearor, Model 985–370, Biospec Products, Inc., Bartlesville, OK, USA), keeping cells cold in an ice-bath. Aliquots of the cell homogenate were kept at −70 °C for assay of thiobarbituric acid reactive substances (TBARS). The remaining cell homogenate was centrifuged for 15 min at 4 °C and 10,000*g*. Supernatant (cytoplasmic fractions) was stored at −70 °C for assays of GSH, GSS protein, and total antioxidants. All assays were performed within one month after the sample collection, except GSS protein immunoassay which was done within 2 months.

2.5. Extracellular fraction collection

Extracellular fractions of HUVEC were collected for the 8-epi-PGF2α immunoassay. The medium of confluent cell culture was collected just before harvesting confluent cells. Samples were stored at −70 °C until the 8-epi-PGF2α immunoassay was performed within one month.

2.6. Nuclear fraction preparation

Nuclear fractions of HUVEC were isolated with a commercial nuclear extraction kit (Cayman Chemical). After 24-h incubation with GGC alone or together with CLA, cells were rinsed, scraped, suspended into ice-cold PBS containing phosphatase inhibitors, and centrifuged for 5 min at 4 °C and 300*g*. Cells were collected from two flasks per each treatment and pooled. Then, cells were suspended and lysed with a hypotonic buffer and 10% (w/v) Nonidet P-40. After spinning, the cell pellet was re-lysed and centrifuged for 10 min at 4 °C and 14,000*g*. Supernatant was collected and stored at −70 °C until transcription factor assays were performed. The assays were done within three days after the sample collection.

2.7. Peroxisome proliferator-activated receptor-γ (PPARγ and nuclear factor-κB (NF-κB) p65 transcription factor assays

Because redox sensitive transcription factors, PPARγ and NF-κB, may play a role in regulating gene expression involved in antioxidant defense (Nakamura and Omaye, 2010), PPARγ and NF-κB p65 DNA binding activities in the nuclear fractions of HUVEC were assessed with PPARγ and NF-κB (human p50/p65 combo) transcription factor assays, respectively (Cayman Chemical). Either human PPARγ bound to PPRE (5'-AGGTCAAAGGTCA-3') or human NF-κB bound to a specific sequence (5'-GGGACTTTCC-3') immobilized within the bottoms of 96 wells was assessed individually at 450 nm with the enzyme-linked immunoassays. All sample tests were replicated ($n = 4$).

2.8. 8-epi $PGF_{2\alpha}$ enzyme immunoassay

8-epi $PGF_{2\alpha}$ is commonly used as a biomarker of oxidative stress along with TBARS (Vincent et al., 2007). Extracellular levels of 8-epi-$PGF_{2\alpha}$ (free 8-epi-$PGF_{2\alpha}$ released into the EGM medium of cell culture) were measured at 405 nm spectrophotometrically with a commercial immunoassay (Cayman Chemical). All sample tests were replicated ($n = 4$).

2.9. Thiobarbituric acid reactive substance (TBARS) assay

Lipid peroxidation as the complex of thiobarbituric acid and malondialdehyde in the cell homogenate of HUVEC was assessed at 535 nm spectrophotometrically. A mixture of thiobarbituric acid, trichloroacetic acid, and hydrochloric acid was added to the cell homogenate, and the mixture was heated for 15 min at 100 °C (Burge and Aust, 1978). The supernatant was collected for reading spectrophotometrically after centrifugation for 10 min at 1000*g*. All sample tests were replicated ($n = 4$).

2.10. Glutathione (GSH) assay

Intracellular GSH levels of HUVEC were determined by the end point method, using a commercial GSH assay (Cayman Chemical), and measured spectrophotometrically at 405 nm. All sample tests were replicated ($n = 4$).

2.11. Total antioxidant assay

Intracellular antioxidant levels of HUVEC were examined with a commercial antioxidant assay (Cayman Chemical). Total antioxidant levels in samples were measured spectrophotometrically at 405 nm. All sample tests were replicated ($n = 4$).

2.12. GSH synthetase (GSS) protein immunoassay

GSS protein levels of HUVEC were detected spectrophotometrically at 450 nm, using rabbit polyclonal antibodies against human GSS (polyclonal; Abcam) and immunoassay reagents (EXTRA3 and SIGMAFAST OPD; Sigma–Aldrich). All sample tests were replicated ($n = 4$).

2.13. RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR)

GSS mRNA levels of HUVEC were assessed by qRT-PCR method. Total RNA was extracted from HUVEC cultured on a 96-well plate (two wells per each treatment) with a Power SYBR® Green Cells-to-CT™ Kit (Invitrogen), and was used as a template for cDNA synthesis with oligodT primers. Reverse transcription reactions were performed for 60 min at 37 °C and inactivated for 5 min at 95 °C. The cDNA was stored for 6 weeks at −20 °C until the qRT-PCR method was performed. The primer sets used to amplify the GSS cDNA were: F-5'-GCAGGCTGATGGTATGGAAT- 3' and R-5'-TACGCCTTTTCTAGGCTCCA-3'. Forty cycles of qRT-PCR reactions were performed for 15 s at 95 °C and for 1 min at 60 °C. Relative expression was calculated from cycle threshold values ($2^{-\Delta\Delta Ct}$ method), using 18S rRNA expression as an internal control for each sample. All sample tests were replicated ($n = 4$).

2.14. Statistical analysis

Statistical analyses (ANOVA, Student's *t*-test, and Pearson's correlations) were performed with SPSS-PASW18. Differences with $p < 0.05$ were considered to be statistically significant. All results were expressed as mean ± standard deviation.

3. Results

3.1. Cell viability

Cytotoxicity, approximately 40% cell death, was microscopically observed in cells treated with GGC and 100 μmol/L CLA after 24 h-incubation. No change in cell viability was detected microscopically in cells with other treatments after the incubation. Consequently, the highest dose of CLA used in experiments reported here was 50 μmol/L.

3.2. Transcription factor DNA binding

Compared to control cells not treated with GGC and CLA, we found significantly higher PPARγ DNA binding levels in cells treated with GGC alone (1.48-fold, $p < 0.005$) or together with 10 μmol/L CLA (1.63-fold, $p < 0.005$) (Fig. 1). Significantly lower levels of PPARγ DNA binding were observed in cells treated with GGC and 50 μmol/L CLA (0.79-fold, $p < 0.05$), compared to cells treated with GGC alone (Fig. 1). The *p*-values of PPARγ DNA binding levels were <0.0001 through one-way ANOVA (Fig. 1).

In contrast to PPARγ DNA binding levels, significantly lower levels of NF-κB p65 DNA binding were found in cells treated with GGC alone (0.64-fold, $p < 0.005$) or together with 10 and 50 μmol/L CLA (0.69-fold, $p < 0.005$ and 0.66-fold, $p < 0.01$, respectively) in comparison to controls (Fig. 2). In addition, statistically higher levels of NF-κB p65 DNA binding were observed in cells treated with GGC and 10 μmol/L CLA (1.08-fold, $p < 0.05$) than those treated with GGC alone, though this small increase is unlikely to be significant physiologically (Fig. 2). NF-κB p65 DNA binding levels showed a statistical significance ($p < 0.0001$) through one-way ANOVA (Fig. 2).

3.3. Oxidative stress biomarkers

Compared to controls, we observed significantly lower levels of 8-epi-$PGF_{2\alpha}$ in cells treated with GGC alone (0.76-fold, $p < 0.01$), whereas cells treated with GGC and 10 μmol/L CLA had 12% higher 8-epi-$PGF_{2\alpha}$ levels than controls ($p < 0.01$)(Fig. 3). Compared to cells treated with GGC alone, higher levels of 8-epi-$PGF_{2\alpha}$ were found in cells treated GGC and either dose of CLA (1.47-fold, $p < 0.005$ and 1.39-fold, $p < 0.01$, respectively) (Fig. 3). 8-epi-$PGF_{2\alpha}$ levels were significant with the *p*-values of <0.0001 through one-way ANOVA (Fig. 3).

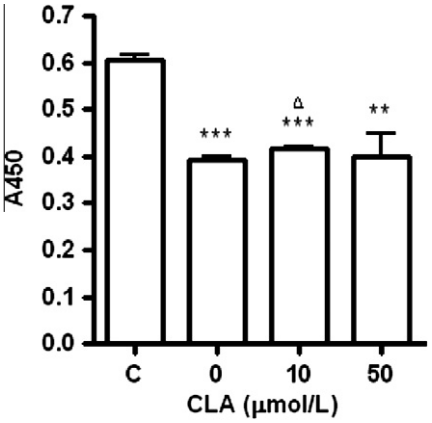

Fig. 2. Nuclear factor-κB (NF-κB) p65 DNA binding levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. $^{**}p < 0.01$ and $^{***}p < 0.005$, compared to control not treated with GGC and CLA through Student's *t*-test. $^{\Delta}p < 0.05$, compared to treatment with GGC alone (100 μmol/L). The *p*-values of one-way ANOVA is <0.0001. All test samples were replicated ($n = 4$).

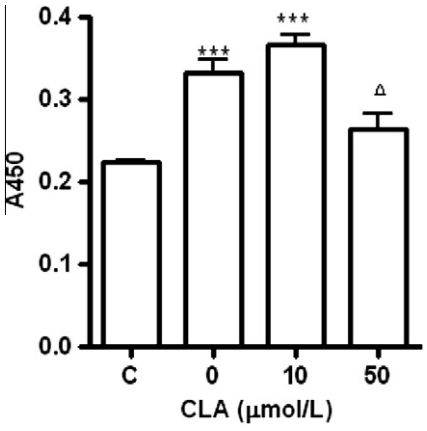

Fig. 1. Peroxisome proliferator-activated receptor γ (PPARγ) DNA binding levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. $^{***}p < 0.005$, compared to control not treated with GGC and CLA through Student's *t*-test. $^{\Delta}p < 0.05$, compared to treatment with GGC alone (100 μmol/L). The *p*-values of one-way ANOVA is <0.0001. All test samples were replicated ($n = 4$).

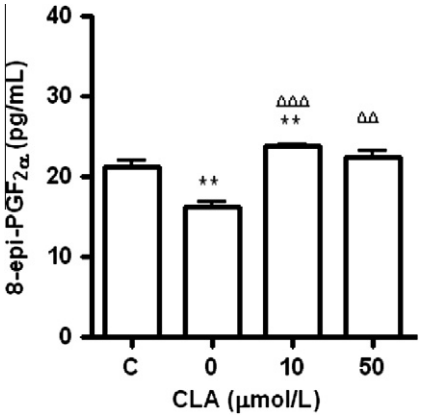

Fig. 3. Extracellular levels of 8-epi-$PGF_{2\alpha}$ after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. $^{**}p < 0.01$, compared to control not treated with GGC and CLA through Student's *t*-test. $^{\Delta\Delta}p < 0.01$ and $^{\Delta\Delta\Delta}p < 0.005$, compared to treatment with GGC alone (100 μmol/L). The *p*-values of one-way ANOVA is <0.0001. All test samples were replicated ($n = 4$).

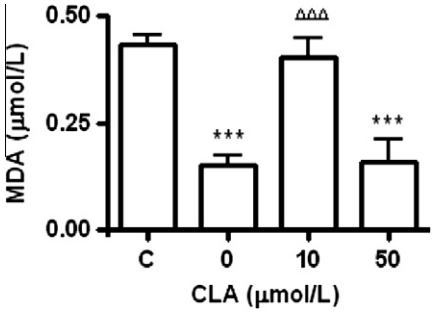

Fig. 4. Thiobarbutric acid reactive substance (TBARS) levels in cell homogenate of human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. $^{***}p < 0.005$, compared to control not treated with GGC and CLA through Student's *t*-test. $^{\Delta\Delta\Delta}p < 0.005$, compared to treatment with GGC alone (100 μmol/L). The *p*-values of one-way ANOVA is <0.0001. All test samples were replicated ($n = 4$).

Compared to controls, we found significantly lower levels of TBARS in cells treated with GGC alone (0.35-fold, $p < 0.005$) or GGC and 50 μmol/L CLA (0.37-fold, $p < 0.005$) (Fig. 4). In contrast, TBARS concentrations were near control levels in cells treated with GGC and 10 μmol/L CLA and were significantly higher (2.67-fold, $p < 0.005$), when compared to cells treated with GGC alone (Fig. 4). The *p*-values of TBARS levels were <0.0001 through one-way ANOVA (Fig. 4).

3.4. Antioxidant levels

No significant changes in total antioxidant levels were found in cells with all treatments, compared to either controls or cells treated with GGC alone (Fig. 5). Treatment with GGC alone did not result in a statistically significant decrease in GSH levels, compared to controls (Fig. 6). In contrast, significantly higher levels of GSH were found in cells treated with GGC and 10 μmol/L CLA, compared to either controls (1.44-fold, $p < 0.01$) or cells treated with GGC alone (1.87-fold, $p < 0.01$) (Fig. 6). However, treatment of cells with GGC and 50 μmol/L CLA resulted in markedly lower GSH levels when compared to either controls (0.3-fold, $p < 0.005$) or cells treated with GGC alone (0.39-fold, $p < 0.005$) (Fig. 6). GSH levels exhibited a statistical significance ($p < 0.0001$) through one-way ANOVA (Fig. 6).

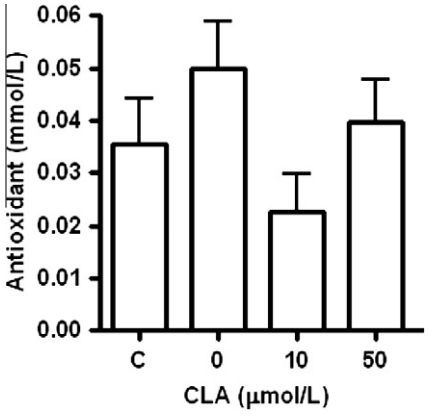

Fig. 5. Total antioxidant levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. No statistical significance was obtained through either Student's *t*-test or one-way ANOVA. All test samples were replicated ($n = 4$).

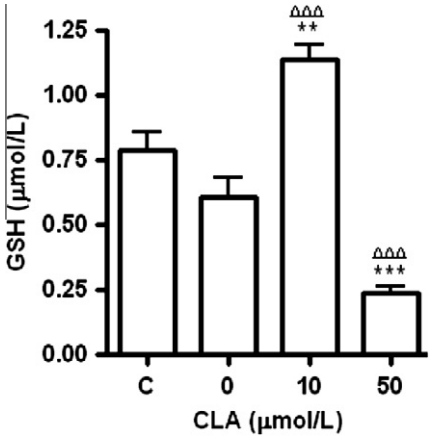

Fig. 6. Glutathione (GSH) levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. $^{**}p < 0.01$ and $^{***}p < 0.005$, compared to control not treated with GGC and CLA through Student's *t*-test. $^{\Delta\Delta\Delta}p < 0.005$, compared to treatment with GGC alone (100 μmol/L). The *p*-values of one-way ANOVA is <0.0001. All test samples were replicated ($n = 4$).

3.5. GSH synthetase (GSS) expression

Although no significant changes in GSS mRNA levels were found in cells with all treatments (Fig. 7), those levels showed a positive correlation trend with PPARγ DNA binding levels ($r = 0.946$, $p = 0.054$). GSS protein levels were 10% lower than controls in cells treated with GGC alone (Fig. 8), while GSS protein levels were 8% higher in cells treated with GGC and either dose of CLA (Fig. 8). These data translated into GSS protein levels being 20% higher in these CLA groups compared to those treated with GGC alone ($p < 0.005$) (Fig. 8). A positive correlation was found between GSS protein and 8-epi-$PGF_{2\alpha}$ levels ($r = 0.972$, $p < 0.05$). GSS protein levels had a statistical significance ($p < 0.0001$) through one-way ANOVA (Fig. 8).

4. Discussion

Higher levels of TBARS, 8-epi-$PGF_{2\alpha}$, GSH, and GSS protein were found in human umbilical vein endothelial cells (HUVEC) treated with 100 μmol/L GGC and 10 μmol/L CLA, compared to treatment with GGC alone, suggesting prooxidant effects of CLA at the low

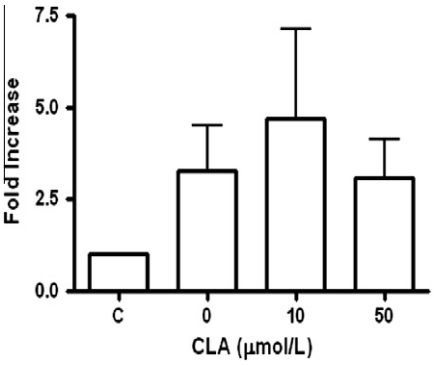

Fig. 7. Glutathione synthetase (GSS) mRNA levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. No statistical significance was obtained through either Student's *t*-test or one-way ANOVA. All test samples were replicated ($n = 4$).

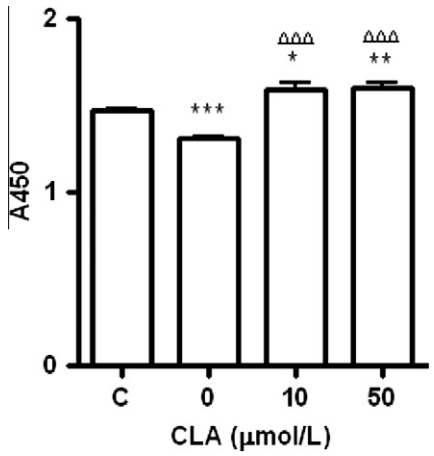

Fig. 8. Glutathione synthetase (GSS) protein levels in human umbilical vein endothelial cells (HUVEC) after 24-h incubation with γ-glutamylcysteine (GGC) alone (100 μmol/L) or combined with conjugated linoleic acid (CLA; 10, 50, or 100 μmol/L). Values are means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$, compared to control not treated with GGC and CLA through Student's *t*-test. $^{\Delta\Delta\Delta}p < 0.005$, compared to treatment with GGC alone (100 μmol/L). The *p*-values of one-way ANOVA is <0.0001. All test samples were replicated ($n = 4$).

dose similar to our previous CLA study. Higher levels of TBARS were observed only in HUVEC treated with low dose of each CLA isomer (5 μmol/L), compared to controls not treated with CLA isomers (Nakamura and Omaye, 2009). Compared to linoleic acid, CLA is more susceptible to oxidation, likely due to their conjugated bonds (van den Berg et al., 1995; Campbell et al., 2003), thus, CLA may be oxidized and serve as a prooxidant at the low dose of 10 μmol/L (i.e., increased exposure of each CLA molecule to oxygen). In our previous GGC study (Nakamura et al., 2012), we found a possible binding site for NF-κB, but none for PPARγ, in the promoter region of GSS gene and lower levels of GSS protein, NF-κB p65 DNA binding, GSH, and oxidative stress, compared to control cells treated without GGC. In the current study, GSH synthesis mediated by oxidative stress involves the pro-inflammatory NF-κB p50/p65 pathway. GSS expression and GSH levels may be induced through the pro-inflammatory NF-κB p50/p65 pathway when oxidative stress (or prooxidant activity of CLA) surpasses the antioxidant capacity of GGC co-administrated and other antioxidant defense. GCS and GSS are involved in GSH synthesis and are inducible (Huang et al., 2000), and exogenous substrates for GSS synthesis (i.e., GGC) are more effective when GSH levels are depleted (Takagi et al., 2010). Thus, GGC may serve as a substitute for GSH likely because of the −SH group in its structure (Grant et al., 1997; Ristoff et al., 2002) in normal or low to mild oxidative stress situations (i.e., 50 μmol/L CLA, see details below) and as a substrate for GSH synthesis under extensive oxidative stress (i.e., 10 μmol/L CLA). However, it is unsure whether NF-κB p65 DNA binding levels were high enough to produce physiological affects despite its small but statistically significant increase in comparison to the treatment with GGC alone. Hence, there is the possibility of other pro-inflammatory mechanisms may be involved in up-regulation of GSS protein and GSH levels at the low dose of CLA. Unexpectedly, cytotoxicity of approximately 40% cell death was observed in cells treated with GGC and 100 μmol/L CLA after 24 h-incubation. Cytotoxicity of CLA, in particular the trans-10, cis-12 CLA isomer, to cancer cells has previously been reported only at low concentrations (5 to 10 μmol/L), but not at high concentrations of the CLA isomer (100 μmol/L total) (Yamasaki et al., 2005). Further investigations are warranted to determine the mechanism of CLA-induced cytotoxicity in interactions between GGC and CLA.

Despite lower levels of GSH, treatment with GGC and 50 μmol/L CLA appears to be protective from oxidative stress similar to treatment with GGC alone, which is indicated by lower levels of TBARS, when compared to control cells not treated with GGC and CLA. GSH levels were even lower in cells treated with GGC and 50 μmol/L CLA than in cells treated with GGC alone, while levels of 8-epi-$PGF_{2\alpha}$ and GSS protein were higher than the treatment with GGC alone and positively correlated. These changes in 8-epi-$PGF_{2\alpha}$ and GSS protein seem not to be related to GSH, TBARS, and NF-κB p65 or PPARγ DNA binding levels. The increase in 8-epi-$PGF_{2\alpha}$ levels was near control concentrations along with low TBARS levels, suggesting the increase was not due to higher free radical or ROS generation. This inconsistency was not seen in our GGC study, indicating it is related to CLA-specific induction. Because CLA (in particular the trans-10, cis-12-CLA isomer) increases free 8-epi-$PGF_{2\alpha}$ levels through competition between CLA and 8-epi-$PGF_{2\alpha}$ for peroxisomal β-oxidation and modulation of its enzyme system activities, the increase in 8-epi-$PGF_{2\alpha}$ levels along with CLA supplementation does not result from increased lipid peroxidation, as suggested by Iannone et al. (Iannone et al., 2009).

Low levels of GSH without increasing oxidative stress observed at the 50 μmol/L dose CLA together with GGC suggest CLA-mediated suppression of GSH synthesis through post-translational modification of GSS (e.g., inhibition of its enzymatic activity) or CLA-induced stability of existing GSS protein and/or GSH degradation. Exogenous GGC may serve as a substitute for GSH under our conditions or in absence of extensive oxidative stress. CLA may also have synergistic antioxidant effects on GGC due to lower levels of GSH compared to GGC treatment. In addition, CLA-induced antioxidative changes observed with the treatment seem to be modulated in a PPARγ independent and NF-κBp50/p65 dependent manner. In fact, CLA has been reported to down-regulate NF-κB p50/p65 activation and the expression of its target gene COX-2 as a ROS generator (Iwakiri et al., 2002; Cheng et al., 2004; Ringseis et al., 2006; Park et al., 2010). Although other investigators have reported that the trans-10, cis-12 CLA isomer induces prooxidative and inflammatory effects through NF-κB p50/p65 activation (Kennedy et al., 2009; Martinez et al., 2010), this does not appear to be a factor at the 50 μmol/L dose used under the conditions of the current study. In contrast, our data at the 10 μmol/L dose of CLA with GGC is consistent with previous reports of prooxidant effects of CLA as mentioned above. Thus, CLA seems to induce 8-epi-$PGF_{2\alpha}$ and existing GSS stability and GSH degradation at the intermediate dose of 50 μmol/L CLA, rather than inducing GSS expression. In our study, both CLA and GGC exhibit differential effects, and the effects depend on doses of CLA or oxidative stress.

5. Conclusions

The results of the present study confirm previous reports that GGC can substitute as an antioxidant for GSH without increasing GSH levels. The efficacy of GGC supplementation in lowering oxidative stress is consistent with our previous findings. Co-administration of CLA with GGC had differential effects depending on dose of CLA in our experimental system. A dose of 100 μmol/L was cytotoxic, whereas a dose of 10 μmol/L seemed to have prooxidant activity without inducing cytotoxicity. In contrast, an intermediate dose of 50 μmol/L CLA with GGC seemed to have antioxidant activity despite a reduction in GSH levels greater than that seen with GGC alone. GGC may play a role not only in GSH synthesis as a substrate but also in protection from oxidative stress as a substitute/antioxidant mediated through a −SH group and as a modulator of GSH synthesis. Due to its ease of permeability through cell membranes, GGC could be used as an intra- and intercellular

therapeutic agent in oxidative stress-related injuries and diseases. Further studies are warranted to develop a better understanding about the efficacy of GGC supplementation under various conditions, for example, in the presence of prolonged/extensive oxidative stress, in either cell culture or animal models. In addition, the value of CLA as an adjunct to GGC to reduce oxidative stress seems to be limited by its narrow range of efficacy. Additional studies with CLA are warranted to better understand its mechanisms of action and which isomers are most effective.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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